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A general cloning strategy for divergent plant cytochrome P450 genes and its application in Lolium rigidum and Ocimum basilicum

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Abstract The investigation of plant cytochrome P450 genes and enzymes is a field of growing interest. Apparently, an even greater diversity of cytochrome P450 genes exists in plants in comparison to other eukaryotes. This may be due to their role in the biosynthesis of secondary metabolites that are present in plants in an enormous variety. Most cloning approaches are hampered by the large sequence diversity of plant cytochrome P450 genes. We present a method to clone divergent cytochrome P450 ESTs by a nested RT-PCR-strategy. These ESTs were used for the subsequent cloning of the corresponding full-size cDNAs of divergent families via cDNA-library screening. Sixteen cytochrome P450 genes belonging to different cytochrome P450-families have been identified in this way, proving the efficacy of the strategy.

Keywords Cloning strategy · ESTs · *Lolium rigidum* · *Ocimum basilicum* · Plant cytochrome P450

Introduction

Cytochrome P450 enzymes are membrane-bound, heme-iron containing enzymes and mono-oxygenases in most cases, that use $NADPH+H^+$ as a co-substrate by forming a complex with a cytochrome P450 reductase. The most-common reactions catalyzed in plants are hydroxylations, but with other reactions like epoxidations (Schuler 1996), oxidative dealkylation (Frear 1995) or phenol-coupling (Stadler and Zenk 1993). The first plant cytochrome P450 that was cloned was a Cyp 71A1 from *Persea americana* (Bozak et al. 1990). This was followed by investigations concerning the role of cytochrome P450 enzymes in many biochemical processes, such as flavonoid biosynthesis (Holton et al. 1993), cyanogenic glucosides (Koch et al. 1995; Bak et al. 1998; Kahn et al. 1999), synthesis of alkaloids (e.g. De-Eknamkul et al. 1992; Kammerer et al. 1994; Nasreen et al. 1996), terpenoids (Hallahan and West 1995; Lupien et al. 1995), de-methylation of steroids (Cabello-Hurtado et al. 1997), hydroxylation of brassinosteroids (Choe et al. 1998), DIMBOA-biosynthesis (Frey et al. 1997) and fatty acid hydroxylation (Cabello-Hurtado et al. 1998; Benveniste et al. 1998).

It is evident that, especially in plants, an enormous diversity of cytochrome P450 genes has evolved (reviewed in Bolwell et al. 1994; Durst and Nelson 1995; Nelson et al. 1996; Schuler 1996; Nelson 1999). This is obviously due to the chemical diversity of secondary metabolites, reflected by the diversity of cytochrome P450 genes that take part in their biosynthesis. For *Arabidopsis thaliana*, a plant not especially rich in secondary metabolites, 273 cytochrome P450 genes that are grouped in 45 families are currently known (Nelson-Lab-Homepage: http://drnelson.utmem.edu/CytochromeP450.html, Febr. 2001). Most of them were identified by the *Arabidopsis* genome sequencing project (The *Arabidopsis* Genome Initiative 2000, further references given therein).

Here we describe an approach for cloning divergently expressed cytochrome P450 genes. This leads to a collection of cytochrome P450 genes from a plant with interesting enzymatic activities. These heterologously expressed enzymes may then be used for studies on substrate specificities. Thus, potentially interesting products or reaction specificities may be identified.

Valuable sources of genes are plants with cytochrome P450-mediated herbicide resistances or plants with many secondary metabolites. *Lolium rigidum* is a grass that has

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developed numerous herbicide resistances in a few years after the first applications. In the line SLR31 it was shown that these cross-resistances to different classes of herbicides are not due to target enzyme changes but to a cytochrome P450-mediated de-toxification (Heap and Knight 1982, 1986, 1990, Holtum et al. 1991; Cotterman et al. 1990; Cotterman and Saari 1992; Powles et al. 1990; Powles and Matthews 1992; Christopher et al. 1991, 1992, 1994; O'Keefe et al. 1991; Zimmerlin and Durst 1992; Burnet et al. 1993 a, b; Hall et al. 1994). As an alternative species *Ocimum basilicum* was chosen for its content of terpenoids (Hegnauer 1966).

Materials and methods

Plant material

Seeds of *L. rigidum* SLR 31 were kindly provided by S.B. Powles (University of Adelaide, Austalia). The *O. basilicum* cultivar Genoveser was provided by H. Jaksch (Institut für Gemüsebau, Weihenstephan, Germany).

mRNA-preparations

For the RNA-preparation from *L. rigidum* SLR 31 all green parts of non-flowering plants with 3–4 leaves were used. From *O. basilicum* cv Genoveser non-flowering apical shoot parts were harvested. The plant material was frozen in liquid nitrogen immediately after harvesting. The mRNA was prepared by using poly(dT)-coated magnetic particles for the specific extraction of mRNA (Dynal, mRNA-Direct-Kit # 610.11). The mRNA was treated with RNAse-free DNAse (Boehringer) which was heat-inactivated afterwards and removed by phenolisation.

Cytochrome P450 ESTs

For the cDNA-synthesis DNA-free RNA was used. The reaction was performed with Superscript Reverse Transcriptase (Gibco BRL) and primed with anchored oligo (dT)-primers. The first RT-PCR was performed with a set of primers directed against the conserved EEF(R)PER-motif in combination with a poly-A-anchored primer. The primers are similiar to the ones published by Frank et al. (1996) except that the five bases at the very 3′-end were maintained non-degenerated by using a set of four primers:

EEF(R)PER-1:
GCGGATCCGA(G/A)GA(G/A)TT(C/T)(A/C)G(G/A/C/T)CC(G/A/C/T)GAGAG,

EEF(R)PER-2:
GCGGATCCGA(G/A)GA(G/A)TT(C/T)(A/C)G(G/A/C/T)CC(G/A/C/T)GAGCG,

EEF(R)PER-3:
GCGGATCCGA(G/A)GA(G/A)TT(C/T)(A/C)G(G/A/C/T)CC(G/A/C/T)GAAAG,

EEF(R)PER-4:
GCGGATCCGA(G/A)GA(G/A)TT(C/T)(A/C)G(G/A/C/T)CC(G/A/C/T)GAACG,
$EcoT_{17}V$: CGGAATTCT$_{17}$(G/A/T).

Each of the four EEF(R)PER-primers was used in combination with the poly-A-anchored primer $EcoT_{17}V$ for the first RT-PCR under the following conditions: 0.4 ng of cDNA, 0.2 mM of dNTPs, 2.5 µM of single EEF(R)PER-Primer, 0.25 µM of $EcoT_{17}V$-Primer, 1 U of *Taq*-Polymerase (Boehringer) in 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris/HCl pH 8.0 with a volume of 40 µl. Cycling conditions were: 30 times for 2 min at 94°C, 2 min at 50°C, 2 at min 72°C; and 5 min at 72°C. These PCR-products were diluted by a factor of 10^3 and used as a template for the second, nested RT-PCR. For this PCR a set of eight non-degenerated primers directed against the PFG-motif (Schopfer and Ebel 1998) as part of the heme-binding-site were used in combination with the poly-A-anchored primers GT_{11}(G/A/C)G, GT_{11}(G/A/C)A, GT_{11}(G/A/C)C, PFG1 (CGCCATTTGG), PFG2 (CGCCATTCGG), PFG3 (CGCCCTTTGG), PFG4 (CGCCCTTCGG), PFG5 (CGCCGTTTGG), PFG6 (CGCCGTTCGG), PFG7 (CGCCTTTTGG), and PFG8 (CGCCTTTCGG). This nested RT-PCR was performed using the following conditions: 5 µl of EEF(R)PER-RT-PCR-product 1:10^3 diluted, 0.2 mM dNTPs, 0.5 µM single PFG-Primer, 2.5 µM of single anchored-Primer, 1 U of *Taq*-Polymerase (Boehringer) in 50 mM KCl, 2 mM $MgCl_2$, 3% DMSO, 10 mM Tris/HCL pH 8.4 with a volume of 40 µl. Cycling conditions were: Hotstart, 40 times for 30 s at 94°C, 2 min at 40°C, 30 s at 72°C; and 5 min at 72°C. The PCR-products were cloned in pUC57/T using the T-cloning-kit (Fermentas, # K1212).

cDNA-library

The mRNA prepared by the magnetic extraction kit was used as starting material. The cDNA-library was established using the λ-ZAP-system (Stratagene, cDNA-Synthesis-Kit # 200401, Gigapack III-Gold-Kit # 200203, Uni-ZAP XR-Vector-Kit # 237211) (Short et al. 1988).

Screening and subcloning

The library screening of the λ-ZAP-cDNA-Library was done using the standard molecular techniques described in Sambrook et al. (1989). The subcloning of the positive λ-phages was performed by the ZAP-protocol as supplied by the manufacturer Stratagene (Short et al. 1988).

Sequencing

For sequencing, fluorescence-labelled ddNTPs were used. The reaction was performed with the DYEnamic ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech Inc., # USB1050). The gels were run on a Abi Prism 377 DNA Sequencer.

Prediction of secondary protein structures

The prediction of secondary protein structures was performed by using the Predict Protein Server (http://www.embl-heidelberg.de/predictprotein/predictprotein.html#PP1SEC).

Results

Cytochrome P450 ESTs

The cloning of cytochrome P450 sequences is hampered by their large sequence diversity. Members of different families show conservation of only a few amino acids and even less motifs are conserved for all families. A first attempt to clone cytochrome P450 ESTs relied on the conserved-sequence motif PFG in the heme-binding region (Schopfer and Ebel 1998). Individual bands from a RT-PCR with the PFG-primers and poly-A-anchored

Fig. 1 Amino-acid sequences of the cytochrome P450-ESTs from *L. rigidum* and *O. basilicum*, positions variable for the different clones of each EST-group are underlined, # indicates a stop-codon. The homologues identified by the BLAST-algorithm of these ESTs are cytochrome P450 sequences from different Cyp-families and various plants

Lolium rigidum-ESTs

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Lol-4/6-G-15      PFGSGRRMCP  GINFATLTFE  IILANLIYHF  DWELPEGSPG
Lol-2/2-C-21      PFGAGRRMCP  GINLATTNIE  LMLANLIYHF  DWELPPGLKR
Lol-3/8-G-59      PFGSGRRICP  GMIFAEANME  IVLATLLYHF  DWELPGGAKV
Lol-3/8-G-29      PFGSGRRICP  GMIFAEANME  IVLATLLYHF  DWELPGGTKV
Lol-1/6-C-34      PFGAGRRICP  GINIGLTNIE  LMLANLIYNF  DWEPSLGVEI
Lol-1/1-G-20      PFGAGRRICP  GLNFGLATVE  IMLALLAYCF  DWELPDGVED
Lol-3/8-G-67      PFGVGRRICP  GIDFAHATIE  MALASLLYHF  DWDLPKGVEV
Lol-4/7-G-20      PFGAGRRICP  GMLFAQSIME  LALAALLYHF  DWELPHGVKP
Lol-1/8-C-141     PFGSGRRMCP  GMVFAEVNME  IVLAALLYHF  NWEIPGGRKP
Lol-4/5-G-31      PFGHGPRICP  GQHLAMVELK  VVLVHLLSKF  AFSPSPGYRH
Lol-1/1-G-41      PFGGGPRICI  GQNFALLEAK  MALSTILQHF  SLELSPSYTH
Lol-4/5-G-46      PFGAGRRACP  GMPMATRSVM  LILASL
Lol-1/6-C-27      PFGMGRRRCP  GETLALRTIG  MVLATLVQCF  DWEPVDGVKV
Lol-3/8-G-148     PFGAGRRMCP  GYSLGMHHAE  YFVARMVMDL  EWRPPVEGEE
Lol-2/5-C-3       PFGSGRRMCP  GSNFGLAALE  LILARFLYYF  NWSLPAGTRP
Lol-4/6-G-5       PFGSGRRMCP  GSNFGLAALD  LILARLLYYF  NWSLLGGMRP
Lol-1/6-C-22      PFGLFPCCCN  QIGVFWNSQV  VWCLFNKL#

Lol-4/6-G-15      VDMTEAFGMD  VHRKENLLLV  PRVAKMV#
Lol-2/2-C-21      KHIDMTEVFG  LTVRREEKLL  LVPKIRI#
Lol-3/8-G-59      EEVLIT#
Lol-3/8-G-29      EEVDMTEKMG  ITIGRKNDLY  MHALVRVPPV#
Lol-1/6-C-34      KDIDMTEVFG  LTICRKEKLL  LIPKVHM#
Lol-1/1-G-20      IDNTEVFGLC  VHRKEKLMLV  PKIHIAV#
Lol-3/8-G-67      EKIDMTEVFG  AKKKKWAI#
Lol-4/7-G-20      EELDMTEQMG  LAVGRKNDLY  LFAKTKVPLD  GTI#
Lol-1/8-C-141     DELDMSEKMG  LTVRRKNDLY  LHASVCVPLV#
Lol-4/5-G-31      APLFRLTIEP  GFGMPLVVTK  LP#
Lol-1/1-G-41      APYTVITLHP  QHGAQIRIKK  I#
Lol-4/5-G-46
Lol-1/6-C-27      DMTEGGGFAI  PKAMPLEAVC  RPRAVMRDVL  QNL#
Lol-3/8-G-148     VDMAEMLDFT  TVIKHPLRAR  LFART#
Lol-2/5-C-3       EELDMDMVVG  ATSKKRKPLN  LIATPYNVSM  KSHS#
Lol-4/6-G-5       DELDTEMIAG  ATAKKKNPLN  LVATPYNVPM  ETQS#
Lol-1/6-C-22
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Ocimum basilicum-ESTs

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Oci-42-1          PFGWGPRTCV  GQMFAIXEAK  TALFMILQRF  SFELSPSYTH
Oci-2/6-V-5       PFGMGRRSCP  GIILALPILG  LIIARLVSNF  EIMPPAGLRE
Oci-1/2-V-15      PFGSGRRVCP  GLALGERMVM  YLLARLLYSF  DWKLPEGEKI
Oci-1/1-V-9       PFGAGRRICP  GAQLGLDMVT  SMIGQLLHRF  DWAPPKGVAP
Oci-2/5-V-30      PFGAGRRVCP  GAQLGIDLAT  SMIGRLLHHF  RWTPPSGVRG

Oci-42-1          APFTIITTQP  QHGAHLTLHR  L#
Oci-2/6-V-5       VDVSEKGGQF  SLHIANHSTI  VFKPIEAL#
Oci-1/2-V-15      DLSETFGIVT  RKTKPLLAIP  TQRPH#
Oci-1/1-V-9       ESIDVAERPG  VVTFMNTPLQ  AVATPRLGPN  FYQRIGVDD#
Oci-2/5-V-30      EEIDMGENPG  TVTYMRTPVE  AVPTLRLPSH  LYKRVAVDI#
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primers were cloned. This procedure turned out to be inefficient since only 1 of 28 cloned PCR-bands was a cytochrome P450 sequence, as confirmed by the presence of further conserved amino acids of the heme-binding region and the homologies identified by the BLAST-algorithm (Altschul et al. 1990). Many of the non-P450 sequences showed BLAST-homologues with the PFG, or related, motifs. To achieve a more-specific amplification a combination of nested RT-PCRs was tested. The conserved motif EEF(R)PER, about 60 amino-acid residues N-terminal of the PFG-motif, was used; the primers are similiar to those employed by Frank et al. (1996). This first RT-PCR resulted in uniformly size-distributed DNA (with faint bands) of up to about 1 kbp.

Table 1 Closest homologues for the ESTs from *L. rigidum* identified by the BLAST-algorithm. The homologies are given in percent identity/similarity. The efficiency of the cloning process and the diversity of sequences identified shows that this nested RT-PCR approach is suitable for general cloning of cytochrome P450 ESTs

Cytochrome P450-EST	Homologous gene from	Cyp-family and homology
Lol-4/6-G15, Lol-2/6-G-1	*Zea mays*	Cyp 71 C 64/79
Lol-2/2-C-21	*Zea mays*	Cyp 71 C 61/81
Lol-3/8-G-59	*Zea mays*	Cyp 71 C 66/77
Lol-3/8-G-29	*Glycine max*	Cyp 71D 60/68
Lol-1/6-C-34, Lol-2/6-G-4	*Lotus japonicus*	Cyp 71D 55/75
Lol-1/1-G-20	*Lotus japonicus*	Cyp 71D 59/74
Lol-3/8-G-67	*Lotus japonicus*	Cyp 71D 60/74
Lol-4/7-G-20	*Glycine max*	Cyp 71D 61/75
Lol-1/8-C-141, Lol-2/8-G-9	*Glycine max*	Cyp 71D 54/69
Lol-4/5-G-31	*Catharanthus roseus*	Cyp 72 42/63
Lol-1/1-G-41	*Catharanthus roseus*	Cyp 72 60/78
Lol-4/5-G-46	*Arabidopsis thaliana*	Cyp 76 C 73/88
Lol-1/6-C-27	*Helianthus tuberosus*	Cyp 81B 53/63
Lol-3/8-G-148	*Arabidopsis thaliana*	Cyp 89 A 61/76
Lol-2/5-C-3	*Sorghum bicolor*	Cyp 99 A 57/72
Lol-4/6-G-5	*Sorghum bicolor*	Cyp 99 A 65/86
Lol-1/6-C-22	None	? –

Table 2 Closest homologues for the ESTs from *L. rigidum* identified by the BLAST-algorithm. The homologies are given in percent identity/similarity

Cytochrome P450-EST	Homologous gene from	Cyp-family and homology
Oci-42-1	*Catharanthus roseus*	Cyp 72 66/81
Oci-2/6-V-5	*Arabidopsis thaliana*	Cyp 73 77/86
Oci-1/2-V-15	*Arabidopsis thaliana*	Cyp 76 C 57/76
Oci-1/1-V-9	*Arabidopsis thaliana*	Cyp 98 A 62/79
Oci-2/5-V-30	*Glycine max*	Cyp 98 A 75/86

This DNA was diluted 1:10^3 and used as a template for the nested PFG-PCR resulting in several discrete fragments also up to about 1 kbp. In most cases different fragments were obtained for different combinations of EEF(R)PER- and PFG-primers. These nested PCR-products were cloned and sequenced. The ESTs clones from *Lolium* and *Ocimum* were predominantly cytochrome P450 sequences, only very few clones were non-P450 sequences. The cytochrome P450 ESTs could be grouped by amino-acid sequence homology (Fig. 1, Table 1, 2); most of these EST-groups are represented by several different clones. For each EST-group only few amino acids were variable for the different EST-clones. Otherwise they differed mainly in the length and sequence of the 3′-untranslated region.

Cytochrome P450 genes

The EST-sequences of the families Cyp 81B (Lol-1/6-C-27), Cyp 71 C (Lol-4/6-G15), Cyp 72 (Lol-4/5-G-31, Lol-1/1-G-41), Cyp 71D (Lol-4/7-G-20), Cyp 89 A (Lol-3/8-G-148) and Cyp 99 A (Lol-2/5-C-3, Lol-4/6-G-5) cloned from *L. rigidum* SLR 31 were used to screen the corresponding cDNA-library. Positive λ-ZAP-clones were subcloned and sequenced. Using this technique 16 cytochrome P450-genes have been identified (Table 3).

The genes show sizes of 506 to 521 amino-acid residues which is within the average range for plant cytochrome P450 genes. Using the BLAST-algorithm all the closest homologs identified were plant cytochrome P450 genes. In most cases the *Lolium*-genes belong to the same Cyp-families as the *Lolium*-ESTs that were used for the corresponding cDNA-screens. For some of the homologs the in vivo function has already been defined. Three similar genes from *Lolium* (*Fhh-t*, *Fhh-v*, *Fhh-y*) show a close BLAST-homology to a fatty acid hydroxylase described from *Helianthus tuberosus* (Cabello-Hurtado et al. 1998) [44/63% (identity, similarity) for *Fhh-t*] and belong to the Cyp 81 family. Another one, *Bxh*, is a homolog of the maize *Bx2*-gene [48/65% (identity, similarity)] which takes part in the DIMBOA-pathway as an indole-hydroxylase (Frey et al. 1997), and is a Cyp 71 family member. *Lol-20-d*, *Lol-20-f* and *Lol-3-k* belong to the Cyp71 family too; *Lol-20-d* and *Lol-20-f* are very close homologs with 99/99% identity/similarity. From the Cyp 72-family two groups of genes were identified, *Lol-31-b* and *Lol-31j*, which group together, with *Lol-22*, *Lol-62*, *Lol-78*, *Lol-79* and *Lol-83* as a second group. Single members of the Cyp 89 family (*Lol-2*) and Cyp 99 family (*Lol-5-v*) were also identified.

Theoretical predictions about the secondary protein structure of the genes were performed with the Predict Protein algorithm (see Materials and methods). The genes of the different Cyp-families showed a remarkable conservation of major secondary structure elements (data not shown).

Table 3 Cytochrome-P450-genes identified from *Lolium rigidum* SLR 31

Lolium rigidum gene, Accession number	Length of cDNA, *Lol*-homologies	Homologous genes (BLAST)
Bxh AF321858	Complete cDNA, 507 aa	Cyp71C4, *Zea mays* *Bx-2*, Indole hydroxylase
Lol-20-d AF321859	Complete cDNA, 510 aa	Cyp71D6, *Solanum chacoense* Cyp71D10, *Glycine max*
Lol-20-f AF321860	Complete cDNA, 507 aa, 3 aa-deletion and 3 aa-substitution to *Lol-20-d*	Cyp71D6, *Solanum chacoense* Cyp71D10, *Glycine max*
Lol-3-k AF321863	Complete cDNA, 506 aa	Cyp 71D7, *Solanum chacoense*
Lol-31-b AF321861	Complete cDNA, 521 aa	Cyp72, *Arabidopsis thaliana, Catharanthus roseus* probable Geraniol-10-hydroxylase
Lol-31-j AF321862	Complete cDNA, 520 aa, 1 aa-deletion and 82 aa-substitutions to *Lol-31-b*	Cyp72, *Arabidopsis thaliana, Catharanthus roseus* probable Geraniol-10-hydroxylase
Lol-22 AF321866	Complete cDNA, 518 aa	Cyp 72A1 *Catharanthus roseus* probable Geraniol-10-hydroxylase
Lol-62 AF321867	Complete cDNA, 518 aa, 74 aa-substitutions to *Lol-22*	Cyp 72A1 *Catharanthus roseus*
Lol-78 AF321868	Complete cDNA, 518 aa, 74 aa-substitutions to *Lol-22*, 5 aa-substitutions to *Lol-62*	Cyp 72A1 *Catharanthus roseus*
Lol-79 AF321869	Complete cDNA, 518 aa, - 5 aasubstitutions to *Lol-22*	Cyp 72A1 *Catharanthus roseus*
Lol-83 AF321870	Complete cDNA, 518 aa,- 5 aasubstitutions to *Lol-22*, - 2 aasubstitutions to *Lol-79*	Cyp 72A1 *Catharanthus roseus*
Fhh-t AF321856	Complete cDNA, 517 aa	Cyp 81B1, *Helianthus tuberosus* Fatty acid hydroxylase
Fhh-v AF321855	Complete cDNA, 517 aa, 9 aa-substitutions to *Fhh-t*	Cyp 81B1, *Helianthus tuberosus* Fatty acid hydroxylase
Fhh-y AF321857	Complete cDNA, 517 aa, 7 aa-substitution to *Fhh-t*	Cyp 81B1, *Helianthus tuberosus* Fatty acid hydroxylase
Lol-2 AF321865	5′-Incomplete cDNA, 499 aa	Cyp 89, *Arabidopsis thaliana*
Lol-5-v AF321864	Complete cDNA, 510 aa	Cyp 99A1, *Sorghum bicolor*

Discussion

Cloning of cytochrome P450-genes

There is a growing interest in the investigation and application of plant cytochrome P450 genes. They play an important role in the biosynthesis of many secondary metabolites of plants. These secondary metabolites may be important as repellents or insecticides in crop plants (e.g. DIMBOA and cyanogenic glucosides) which conversely may be deleterious to human consumption. Other secondary metabolites synthesized by cytochrome P450 enzymes are of pharmaceutical importance (e.g. alkaloids). Cytochrome P450 genes are also important for the de-toxification of herbicides. Genes with such functions may lead to valuable transgenic yeasts and plants. Many different methods have been applied to clone cytochrome P450-genes of known function: differential display (e.g. Schopfer and Ebel 1998), tagging (Frey et al. 1997), protein purification (e.g. Koch et al. 1995), cloning via ESTs of predominantly expressed candidate genes and subsequent testing of the heterologously expressed genes with substrates (Holton et al. 1993). The recently finished sequencing of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative 2000) resulted in 273 cytochrome P450 genes. For only a few of them has an enzymatic function been identified (Nelson-Lab-Homepage: http://drnelson.utmem.edu/CytochromeP450.html). These sequences could be used for cloning approaches, either as heterologous probes for library screening or for the design of degenerated primers for PCR-amplification. However, both approaches would probably be restricted to the isolation of genes belonging to the same, or closely related, Cyp-families.

Generally, cloning approaches based on homology are hampered by the large sequence diversity of plant cytochrome P450 sequences and are thus restricted to close homologs. The cloning of divergent sequences is a strategy that could deliver candidate sequences for genes with known functions. It could also form the basis of an

approach where these heterologously expressed genes are used to screen potential substrates in order to identify reactions of interest. Such a cloning approach has to rely on the few conserved amino-acid motifs. For this reason the previously described method was developed to obtain cytochrome P450-ESTs.

In a first approach the PFG-primers were used for a RT-PCR together with a poly-A-tail-anchored primer. From 28 PCR-bands cloned only one turned out to represent a cytochrome P450-sequence. Some others had BLAST-homologues which showed PFG, or related, motifs. This indicates that the PFG-primers are too unspecific to exclusively amplify cytochrome P450 sequences. When additional criteria like differential expression are available this RT-PCR is very useful (Schopfer and Ebel 1998). A better specifity was achieved by the combined use of the PFG- and the more N-terminal EEF(R)PER motifs for primer-design, together with poly-A-tail-anchored primers. This nested-RT-PCR results in ESTs-clones that are almost exclusively cytochrome P450-sequences. Thus the specifities of the two primers can be combined to avoid amplification of non-cytochrome P450-sequences. The ESTs cloned from both *L. rigidum* and *O. basilicum* turned out to be cytochrome P450-sequences from different families and subfamilies.

Screening of a cDNA-library of *L. rigidum* resulted in genes that are members of the same family of cytochrome P450 sequences as the corresponding EST-clone in most cases. In one case screening with a hybridisation probe of the family Cyp 99 (Lol-2/5-C-3) resulted in the isolation of a Cyp 71D gene (*Lol-3-k*). The genes identified with an EST may be very close homologs (e.g. *Lol-20-d*, *Lol-20-f* and *Lol-22*, *Lol-79*, *Lol-83*). Very closely homologous genes could be alleles since a single population was used as starting material. This population had been inbred for some generations but is not strictly isogenic. However, they could alternatively represent a family of orthologous genes with diverse functions. The multiple herbicide resistences of *Lolium rigidum* SLR31 may have occurred by the selection of single members of a group of closely related cytochrome P450 genes. For *A. thaliana* a diversity of 273 cytochrome P450 genes was found (Nelson-Lab-Homepage). The method to produce cytochrome P450 ESTs by a nested RT-PCR-strategy relies on the presence of the conserved motifs EEF(R)PER and PFG. These sequences, or very related ones, are part of at least half of all plant cytochrome P450-sequences already known (Nelson-Lab-Homepage). Therefore the approach described is an efficient way of cloning divergent cytochrome P450 sequences.

Perspectives

Compared to the number of cytochrome P450 sequences already known, and even more to the diversity of genes that can be expected, only few in vivo functions have yet been identified; many cloned plant cytochrome P450 genes are without any known function. These sequences are from the *Arabidopsis* genome sequencing or side-products of cytochrome P450 research. Using methods directed for the cloning of divergent sequences many genes may be isolated from plants with promising properties and without the availability of genomic sequence information. The de-toxification of xenobiotics, like herbicides, shows that plant cytochrome P450 enzymes may possess a broader substrate specifity and can catalyze additional reactions with artificial substrates. Many useful in vivo and in vitro reactions can be expected.

The heterologous expression in the well-established yeast-system co-expressing cytochrome P450 reductases allows one to perform the enzymatic reactions in vitro. Siminszky et al. (1999) have used the system to test heterologously expressed soybean cytochrome P450 enzymes on herbicides. A Cyp71A10 enzyme was shown to hydroxylate phenylurea herbicides. By cloning of a broad spectrum of enzymes, expression libraries can be established. Such collections of heterologously expressed human cytochrome P450 genes have been used to study pharmacological and toxicological problems (Sengstad and Paladino 1997). Other types of genes have also been studied by such expression libraries (Martzen et al. 1999).

For the cytochrome P450 expression libraries it would be of great advantage to make use of the large collections of chemical substances available in the pharmaceutical industry. Automated methods could be used to screen for positive enzymatic tests on these substance libraries. In this way artificial substrates could be identified and potentially valuable reactions be defined. This could lead to an application of the heterologously expressed cytochrome P450 enzyme.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410

Bak S, Kahn RA, Nielsen HL, Möller BL, Halkier BA (1998) Cloning of three A-type cytochromes P450, CYP71E1, CYP98, and CYP99 from *Sorghum bicolor* (L.) Moench by a PCR approach and identification by expression in *Escherichia coli* of CYP71E1 as a multifunctional cytochrome P450 in the biosynthesis of the cyanogenic glucoside dhurrin. Plant Mol Biol 36: 393–405

Benveniste I, Tijet N, Adas F, Philipps G, Salaün J-P, Durst F (1998) CYP86A1 from *Arabidopsis thaliana* encodes a cytochrome P450-dependent fatty acid omega-hydroxylase. Biochem Biophys Res Commun 243: 688–693

Bolwell GP, Bozak K, Zimmerlin A (1994) Plant cytochrome P450. Phytochemistry 37: 1491–1506

Bozak KR, Yu H, Sirevaeg R, Christoffersen RE (1990) Sequence analysis of ripening-related cytochrome P450 cDNAs from avocado fruit. Proc Natl Acad Sci USA 87: 3904–3908

Burnet MWM, Loveys BR, Holtum JAM, Powles SB (1993a) A mechanism of chlorotoluron resistance in *Lolium rigidum*. Planta 190: 182–189

Burnet MWM, Loveys BR, Holtum JAM, Powles SB (1993b) Increased detoxification is a mechanism of simazine resistance in *Lolium rigidum*. Pestic Biochem Physiol 46: 207–218

Cabello-Hurtado F, Zimmerlin A, Rahier A, Taton M, DeRose R, Nedelkina S, Batard Y, Durst F, Pallett KE, Werck-Reichhart D (1997) Cloning and functional expression in yeast of a cDNA coding for an obtusifoliol 14-alpha-demethylase (CYP51) in wheat. Biochem Biophys Res Commun 230: 381–385

Cabello-Hurtado F, Batard Y, Salaün, J-P, Durst F, Pinot F, Werck-Reichart D (1998) Cloning, expression in yeast, and functional characterization of CYP81B1, a plant cytochrome P450 that catalyzes in-chain hydroxylation of fatty acids. J Biol Chem 273: 7260–7267

Choe S, Dilkes BP, Fujioka S, Takatsuto S, Sakurai A, Feldmann KA (1998) The DWF4 gene of *Arabidopsis* encodes a cytochrome P450 that mediates multiple 22-alpha-hydroxylation steps in brassinosteroid biosynthesis. Plant Cell 10: 231–244

Christopher JT, Powles SB, Liljegren DR, Holtum JAM (1991) Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). II. Chlorsulfuron resistance involves a wheat-like detoxification system. Plant Physiol 95: 1036–1043

Christopher JT, Powles SB, Holtum JAM (1992) Resistance to acetolactate synthase-inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. Plant Physiol 100: 1909–1913

Christopher JT, Preston C, Powles SB (1994) Malathion antagonizes metabolism-based chlorsulfuron resistance in *Lolium rigidum*. Pestic Biochem Physiol 49: 172–182

Cotterman JC, Saari LL (1992) Rapid metabolic inactivation is the basis for cross-resistance to chlorsulfuron in Diclofop-Methyl-resistant rigid ryegrass (*Lolium rigidum*) Biotype SR4/84. Pestic Biochem Physiol 43: 182–192

Cotterman IC, Saari LL, Smith, WF (1990) Rapid metabolic interaction: a basis for resistance to chlorsulfuron in annual ryegrass (*Lolium rigidum* Gaud.). Abstr Weed Sci Soc Amer 30: 56

De-Eknamkul W, Tanahashi T, Zenk MH (1992) Enzymic 10-hydroxylation and 10-O-Methylation of dihydrosanguarine in dihydrochelirubine formation by *Eschscholtzia*. Phytochemistry 31: 2713–2717

Durst F, Nelson DR (1995) Diversity and evolution of plant P450 and P450-reductases. Drug Metab Drug Interact 12: 189–206

Frank MR, Deyneka JM, Schuler MA (1996) Cloning of wound-induced cytochrome P450 monooxygenases expressed in *Pea*. Plant Physiol 110: 1035–1046

Frear DS (1995) Wheat microsomal cytochrome P450 monooxygenases: characterization and importance in the metabolic detoxification and selectivity of wheat herbicides. Drug Metab Drug Interact 12: 329–357

Frey M, Chomet P, Glawischnig E, Stettner C, Grün S, Winklmair A, Eisenreich W, Bacher A, Meeley RB, Briggs SP, Simcox K, Gierl A (1997) Analysis of a chemical plant defense mechanism in grasses. Science 277: 696–699

Hall LM, Holtum JAM, Powles SB (1994) Mechanisms responsible for cross resistance and multiple resistance. In: Powles SB, Holtum JAM (eds) Herbicide resistance in plants: biology and biochemistry. Lewis, Boca Raton, pp 243–261

Hallahan DL, West JM (1995) Cytochrome P-450 in plant/insect interactions: geraniol-10-hydroxylase and the biosynthesis of iridoid monoterpenoids. Drug Metab Drug Interact 12: 369–382

Heap I, Knight R (1982) A population of ryegrass tolerant to the herbicide Diclofop-methyl. Aust J Agric Res 48: 156–157

Heap I, Knight R (1986) The occurence of herbicide cross-resistance in a population of annual ryegrass, *Lolium rigidum*, resistant to Diclofop-methyl. Aust J Agric Res 37: 149–156

Heap I, Knight R (1990) Variation in herbicide cross-resistance among populations of annual ryegrass, *Lolium rigidum* resistant to Diclofop-methyl. Aust J Agric Res 41: 121–128

Hegnauer R (1966) Chemotaxonomie der Pflanzen, Band IV. In: Lehrbücher und Monographien aus dem Gebiete der exakten Wissenschaften, Chemische Reihe 19. Birkhäuser Verlag, Basel Boston Stuttgart, pp 312–316

Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, Menting JGT, Lu C-Y, Farcy E, Stevenson TW Cornish EC (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. Nature 366: 276–279

Holtum JAM, Matthews JM, Häusler RE, Liljegren DR, Powles SB (1991) Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). III. On the mechanism of resistance to Diclofop-Methyl. Plant Physiol 97: 1026–1034

Kahn RA, Fahrendorf T, Halkier BA, Moeller BL (1999) Substrate specifity of the cytochrome P450 enzymes Cyp79A1 and Cyp71E1 involved in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. Arch Biochem Biophys 363: 9–18

Kammerer L, De-Eknamkul W, Zenk MH (1994) Enzymic 12-hydroxylation and 12-O-methylation of dihydrochelirubine in dihydromacarpine formation by *Thalictrum bulgaricum*. Phytochemistry 36: 1406–1416

Koch BM, Sibbesen O, Halkier BA, Svendsen I, Moller BL (1995) The primary sequence of cytochrome P450tyr, the multifunctional N-hydroxylase catalyzing the conversion of L-tyrosine to p-hydroxyphenylacetaldehyde oxime in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. Arch Biochem Biophys 323: 177–186

Lupien S, Karp F, Ponnamperuma K, Wildung M, Croteau R (1995) Cytochrome P450 limonene hydroxylases of *Mentha* species. Drug Metab Drug Interact 12: 245–260

Martzen MR, McCraith SM, Spinelli SL, Torres FM, Fields S, Grayhack EJ, Phizicky EM (1999) A biochemical approach for identifying genes by the activity of their products. Science 286: 1153–1155

Nasreen A, Rueffer M, Zenk MH (1996) Cytochrome P-450-dependent formation of isoandrocymbine from autumnaline in colchicine biosynthesis. Tetrahedron Lett 37: 8161–8164

Nelson DR (1999) Cytochrome P450 and the individuality of species. Arch Biochem Biophys 369: 1–10

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6: 1–42

O'Keefe DP, Lenstra R, Omer CA (1991) An enzymatic basis for herbicide resistance: cytochrome P450 monooxygenases. In: Shewry PR, Gutteridge S (eds) Plant protein engineering. Edward Arnold, London, pp 281–291

Powles SB, Matthews JM (1992) Multiple herbicide resistance in annual ryegrass (*Lolium rigidum*): a driving force for the adoption of integrated weed management. In: Denholm I, Devonshire A, Holloman D (eds) Achievements and developments in combating pest resistance. Elsevier, London, pp 75–87

Powles SB, Holtum JAM, Matthews JM, Liljegren DR (1990) Herbicide cross-resistance in annual rye-grass (*Lolium rigidum* Gaud.). The search for a mechanism. In: Green MB, Moberg WK, LeBaroneds H (eds) Managing resistence to agrochemicals. American Chemical Society Symposium Series 421, ACS, Washington D.C., pp 394–406

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA

Schopfer CR, Ebel J (1998) Identification of elicitor-induced cytochrome P450 s of soybean (*Glycine max* L.) using differential display of mRNA. Mol Gen Genet 258: 315–322

Schuler MA (1996) Plant cytochrome P-450 monooxygenases. Crit Rev Plant Sci 15: 235–284

Sengstag C, Paladino G (1997) A collection of *Saccharomyces cerevisiae* strains as an unlimited source of human enzymes. Bioworld 6: 2–7

Short JM, Fernandez JM, Sorge JA, Huse WD (1988) Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res 16: 7583–7600

Siminszky B, Corbin FT, Ward ER, Fleischmann TJ, Dewey RE (1999) Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides. Proc Natl Acad Sci USA, 96: 1750–1755

Stadler R, Zenk MH (1993) The purification and characterization of a unique cytochrome P-450 enzyme from *Berberis stolonifera* plant cell cultures. J Biol Chem 268: 823–831

The *Arabidopsis* Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815

Zimmerlin A, Durst F (1992) Aryl hydroxylation of the herbicide Diclofop by a wheat cytochrome P-450 monooxygenase. Plant Physiol 100: 874–881